Biochemical Genetic Analysis of Indanocine Resistance in Human Leukemia[1]

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ABSTRACT

Indanocine is a potent tubulin-binding drug that is cytotoxic to multidrug-resistant cancer cell lines. We demonstrated that indanocine specifically induces apoptosis in malignant B cells from patients with chronic lymphocytic leukemia. To address the exact biochemical basis for indanocine toxicity, an indanocine-resistant clone was selected from mutagenized CEM human lymphoblastoid cells. The resistant cells displayed a stable indanocine-resistant phenotype for at least 9 months in drug-free culture. The cloned cells are cross-resistant to colchicine and vinblastine, but not to paclitaxel, and do not have increased expression of the multidrug-resistant p170 glycoprotein. In both parental cells and cell extracts, indanocine treatment caused tubulin depolymerization. In contrast, the tubulin in the resistant clone did not depolymerize under identical conditions. Both extract mixing and cell fusion experiments suggested that a stable structural change in microtubules, rather than a soluble factor, was responsible for indanocine resistance. Sequence analysis of parental and resistant cells revealed a single point mutation in the M40 isotype of β-tubulin at nucleotide 1050 (G→T, Lys^{350}→Asn) in the indanocine-resistant clone, in a region close to the putative colchicine binding site.

INTRODUCTION

Indanocine is a potent microtubule-depolymerizing agent with antiproliferative activity (1, 2). Indanocine induces apoptotic cell death in a broad range of human tumor cell lines, including several types of multidrug-resistant cells (2). Microtubules are vitally important for cellular functions such as motility, mitosis, and secretion. In cell-free assays using purified tubulin, we showed that indanocine competes with colchicine binding (2), suggesting that the binding sites for indanocine and colchicine are related to each other.

Indanocine is different from many other microtubule-disrupting drugs, because it displays toxicity toward multidrug-resistant cells and kills nondividing or quiescent cells (2). These results suggest that indanocine might work through a mechanism independent of mitotic arrest. Treatment of human malignancies with antitumor agents often results in acquired resistance, which represents a major limitation to chemotherapy. Resistance to the tubulin-binding antimitotic drugs has been shown to be mediated by increased expression of a drug efflux pump, such as Pgp170 (3), by detoxification of the drug in the cell, and by reduced drug influx (4). In addition to these mechanisms, mutational alterations in β-tubulin have been found in paclitaxel- and epothilone-resistant cell lines (5, 6).

To understand the mechanism of indanocine toxicity, an indanocine-resistant clone was derived from the highly sensitive human T-lymphoblastoid CEM cell line. The resistant line displayed weak cross-resistance to colchicine and vinblastine but remained sensitive

to paclitaxel. Compared with parental cells, the resistant cells exhibited defective indanocine-driven tubulin depolymerization, both in intact cells and cell-free extracts. The resistant cells had a single point mutation in β-tubulin cDNA that caused a Lys to Asn change at position 350, near the putative colchicine-binding site. These results suggest that altered tubulin structure is one primary cause of indanocine resistance.

MATERIALS AND METHODS

Materials. Indanocine structure is shown in Fig. 2D. The compound was synthesized as described (7). Paclitaxel, vinblastine sulfate, colchicine, fludarabine, doxorubicin, staurosporine, and cytochalasin B were purchased from Calbiochem (San Diego, CA) and Sigma Chemical Co. (St. Louis, MO). Unless otherwise designated, all antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz Biotechnology, Santa Cruz, CA).

Primary Lymphocyte Assays. This study was approved by the Institutional Review Board of the University of California, San Diego, and all patients gave informed written consent to participation. Patients had to have B-CLL[3] according to National Cancer Institute criteria of any Rai stage. Peripheral blood from CLL patients or normal donors was layered on top of Ficoll-Paque Plus (Pharmacia, Peapack, NJ) and centrifuged at 1200 × g for 20 min with break off. The enriched PBMCs were washed three times with Ca^{2+}, Mg^{2+}-free HBSS. Normal B cells were purified from buffy coats using the RosetteSep human B cell kit (StemCell Technologies, Inc., Vancouver, British Columbia, Canada) per the manufacturer's instructions. All primary cells were cultured in RPMI 1640 with 20% fetal bovine serum at a density of 2–5 × 10^6 cell/ml. Freshly isolated CLL cells or normal lymphocytes with viability exceeding 95% or frozen CLL cells with viability exceeding 75% at the time of incubation were used. Because CLL cells display a variable degree of spontaneous apoptosis and for the measurement of the IC$_{50}$ of indanocine at 24 h of incubation, the control cells (treated with vehicle only) were normalized at 100%. With freshly isolated lymphocytes, the viability of the controls at 24 h always exceeded 85%, whereas with the frozen CLL samples the mean viability was 65% (range, 45–85%).

Measurement of Lymphocyte Viability. Lymphocyte viability was assessed by enumeration of cells that excluded erythrosin B. In addition, the percentage of viable cells was assessed by flow cytometry. Briefly, cells were incubated for 10 min at 37°C in culture medium containing 40 nM DiOC$_6$ (Molecular Probes, Eugene, OR) and 5 μg/ml propidium iodide (Molecular Probes), followed by analysis in a Becton Dickinson FACScalibur cytofluorometer. After suitable compensation, fluorescence was recorded at different wavelengths, DiOC$_6$ at 525 nm (FL-1) and PI at 600 nm (FL-3). Viable cells were DiOC$_6$-bright and PI-low.

Cell Culture. The human T-lymphoblastoid CEM cell line came from American Type Culture Collection (CRL-119; Rockwell, MD) and was propagated according to the supplier's instructions in RPMI 1640 supplemented with 10% fetal bovine serum. To select for indanocine-resistant clones, 5 × 10^6 CEM cells were cultured for 18 h in the same medium containing 1 mg/ml of the mutagen ethyl methanesulfonate (Sigma Chemical Co.). Ethyl methanesulfonate was then washed out, and the cells were cultured for 2 days in regular medium. After that the cells were put into medium containing 10 nM indanocine. The concentration of indanocine was raised gradually to keep

[3] The abbreviations used are: CLL, chronic lymphocytic leukemia; PBMC, peripheral blood mononuclear cell; DiOC$_6$, 3,3'-dihexyloxacarbocyanine iodide; PI, propidium iodide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Pgp170, P-glycoprotein 170.

Table 1 *Differential sensitivity of normal and malignant B cells to indanocine*

Freshly isolated CLL and normal cells (viability ≥95%) or frozen CLL cells (viability ≥75%) were incubated with increasing concentrations of indanocine ranging from 0.1 to 10 μM for 24 h. The numbers and viabilities of the treated cells were assessed by microscopy and by flow cytometric analysis as described in "Materials and Methods." The IC_{50} is the drug concentration that kills ~50% of the cells. Because CLL cells display a variable degree of spontaneous apoptosis and for the measurement of the IC_{50} of indanocine at 24 h of incubation, the control cells (treated with vehicle only) were normalized to 100%.

Cell type	No. of samples	IC_{50} (μM)
CLL	16	<1
CLL	10	Between 1 and 10
Normal PBMCs	5	>10
Normal B	3	>10

Table 2 *Resistance profile of parental CEM and indanocine-resistant CEM-178 cells*

CEM and CEM-178 cells were continuously exposed to various drugs at serial-diluted concentrations for 72 h. Viable cells were quantified using the MTT assay. The IC_{50} is the drug concentration that inhibited cell growth by 50%.

Drugs	CEM IC_{50}	CEM-178 IC_{50}	CEM:CEM-178
Indanocine	1.79 nM	206 nM	115
Paclitaxel	0.5 nM	0.5 nM	1
Colchicine	2.0 nM	62.5 nM	31
Vinblastine	0.1 nM	4.0 nM	40
Fludarabine	5.8 μM	13.3 μM	2.3
Doxorubicin	0.2 μM	0.4 nM	1.9
Cytochalasin B	7.1 μM	4.6 μM	0.6

viable cells at ~10% of control. The selection was stopped when the resistant variant was able to grow in 300 nM indanocine. The cells were cloned by limiting dilution to yield CEM-178.

Assessment of Cell Growth. The cells were incubated with various concentrations of drugs for 72 h in 96-well plates. Viable cells were quantified by MTT assay, as described (2). The IC_{50} was defined as the concentration of drug required to inhibit cell growth by 50%.

Cellular Assay for Caspase Activity. At the indicated time points, the cells were washed twice with PBS, and the pellets were resuspended in ice-cold caspase buffer [50 mM HEPES (pH 7.4), 100 mM NaCl, 1 mM EDTA, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, and 5 mM DTT] and incubated for 10 min on ice. The samples were then centrifuged at 4°C. Supernatant containing 10–20 μg of total protein was aliquoted into 96-well plates. Fifty μl of hypotonic extraction buffer (HEB, contains 50 mM PIPES, 50 mM KCl, 5 mM EGTA, 2 mM MgCl$_2$, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride) was mixed with lysates. The reactions were initiated by addition of 100 μM of the specific substrate (Ac-DEVD-AMC for caspase-3 and Ac-LEHD-AFC for caspase-9) acquired from Calbiochem. After 1-h incubation at 37°C, caspase activities were measured by monitoring the released fluorescence of AFC or AMC at excitation and emission wavelengths of 400 and 505 nm, and 380 and 460 nm, respectively.

Measurement of Apoptosis by Cytochrome *c* Release. An early event in apoptosis induced by internal factors is the release into the cytosol of mitochondrial cytochrome *c* (8). Cytosolic and mitochondria cytochrome *c* were separated by extracting cells in HEB buffer (see above) freshly supplemented with 0.1% digitonin (Sigma Chemical Co.). The lysates were vortexed briefly and incubated on ice for 10 min, followed by centrifugation at 16,000 × *g* at 4°C for 5 min. The supernatants and pellets represented cytosolic and mitochondria-associated fractions, respectively. The fractions were then analyzed by Western blotting using anti-cytochrome *c* antibody (PharMingen, San Diego, CA).

Tubulin Polymerization Assay. To quantify tubulin polymerization and depolymerization, an assay was developed by modifying the method of Giannakakou *et al.* (5). For each data point, 2 × 10⁶ of cells were incubated in 2 ml of regular medium containing drugs for various periods of time. The cells were then washed twice at room temperature and resuspended in 50 μl of tubulin extraction buffer (1 mM MgCl$_2$, 2 mM EGTA, 0.5% NP40, and 20 mM Tris-HCl, pH 6.8) supplemented with 2 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail (Sigma Chemical Co.). After a brief but vigorous vortex, the lysates were incubated at room temperature for 5 min and then centrifuged at 16,000 × *g* for 5 min to separate the soluble from

polymerized tubulin. The supernatant and pellet fractions were resolved on 10–20% pre-cast Tris-glycine gels (Novex, San Diego, CA) and then subjected to Western blotting with a specific anti-α-tubulin antibody.

Mixing Experiments. Parental CEM cells and the indanocine-resistant clone (CEM-178) were first incubated with 5 μM paclitaxel for 1 h. The cells were then pelleted, washed twice with PBS, and lysed in tubulin extraction buffer. The lysates from CEM and CEM-178 cells were mixed at various proportions. Indanocine (200 μM) was added to the mixed lysate, and the reactions were incubated for 2 h at 37°C. The samples were then spun at 16,000 × *g* for 5 min. Supernatant and pellet fractions were collected and tested by Western blotting.

Cell Fusion Experiment. A hypoxanthine phosphoribosyltransferase-negative and ouabain-resistant CEM cell line was selected as described previously (9). For the fusion experiments, 40 × 10⁶ each of the ouabain-resistant CEM and indanocine-resistant CEM-178 lymphoblasts were mixed in a ratio of 1:1 in serum-free RPMI 1640. Cells were pelleted and incubated for 2 min at 37°C, in 1 ml 50% polyethylene glycol 1500 (PEG 1500; Boehringer Mannheim, Germany) as a fusing agent. Then 1 ml of serum-free RPMI 1640 was dropwise added to the cells at 37°C over 1 min, followed by 7 ml of serum-free RPMI 1640 centrifugation and resuspension at a concentration of 2.5 × 10⁶ cells/ml in regular growth medium. Subsequently, 50 μl of cells were plated in a 96-well plate; the next day, 50 μl of HAT medium (Sigma Chemical Co.) containing 1 μM ouabain (Calbiochem) were added to the cells for double selection. When the cells started to grow, hybridomas resulting from the fusions were selected by limiting dilutions.

Sequence Analysis of Expressed β-Tubulin in CEM-178. Total RNA was extracted from both CEM and CEM-178 using Trizol reagents (Life Technologies, Inc., Gaithersburg, MD) and digested with RNase-free DNase I to eliminate any possible DNA contaminant. cDNA was synthesized by reverse transcription using the Superscript II protocol, followed by RNase H digestion (Life Technologies, Inc.). The cDNA was used as template in PCR reactions with primers specific for the b2 (B1F and B2R) and M40 (M1F and M2R) isotypes of β-tubulin (GenBank accession numbers X02344 and J00314, respectively). The sequences of the primers are shown in Table 3. The PCR was carried out under the following conditions: initial denaturation of 94°C for 12 min, 35 cycles of 94°C 30 s, 48°C (55°C for b2 primers) 30 s, 72°C 80 s, with a final extension at 72°C for 10 min. The resulting 1.2-kb products spanned the full length of the b2 and M40 coding region, except for 17 bp from the 5' and 3' ends. The PCR products were purified using Qiagen kits and sequenced by a core facility (The Scripps Research Institute, La Jolla, CA) using the overlapping primers as illustrated in Fig. 5. To avoid PCR errors introduced by Taq polymerase, each PCR reaction was repeated at least once. All of the sequences were verified in both directions.

RESULTS

Differential Sensitivity of Normal and Malignant B Cells to Indanocine. Because it has been reported previously that CLL cells but not normal lymphocytes are highly susceptible to microtubule-binding agents (10), we tested the short-term (24-h) toxicity of in-

Table 3 *Primers used in sequencing b2 and M40 β-tubulin isotypes*

Primer	Sequence
B1F	TTG CAG GCC GGG CAG TG
B2F	AGT GGT GCT GGG AAC AAC T
B3F	CTC GTA GAA AAC ACA GAC GA
B4F	CAA ATG CTT AAT GTC CAA AAC AAA
B5F	CAT GAA CAC GTT TAG TGT GG
B1R	TCG TCT GTG TTT TCT ACG AG
B2R	GGT ACT CGG ACA CCA GGT CA
B3R	CTG ACC GAA AAC GAA GTT GT
B4R	GAG TGG GTC AGC TGG AAA C
M1F	ATC CAG GCT GGT CAA TG
M2F	TCT GGG GCA GGT AAC AAC T
M3F	TTG GTA GAG AAT ACT GAT GA
M4F	CAG ATG CTT AAC GTG CAG AAC AAG
M1R	TCA TCA GTA TTC TCT ACC AA
M2R	GAT ACT CAG AGA CGA GGT CG
M3R	ACC TGT GGC TTC ATT GTA GT

danocine using freshly isolated and frozen cells from 26 CLL patients and from 8 healthy volunteers. All CLL cells were sensitive to indanocine, with IC_{50}s <10 μM. No differences in indanocine sensitivity were encountered between freshly isolated or frozen CLL cells, nor was there a correlation between the indanocine cytotoxicity and the rate of spontaneous apoptosis. In contrast, normal PBMCs or purified normal B cells were resistant to indanocine at concentrations as high as 10 μM (Table 1). The selective cytotoxicity of indanocine observed in CLL cells was attributable to apoptosis, as indicated by subdiploid DNA content, caspase-3 activation, and cytochrome c release (data not shown). The results obtained with CLL cells pressed us to study further the mechanism of action of indanocine, by developing indanocine-resistant cells.

Characterization of Indanocine-resistant Lymphoblasts. Compared with parental CEM cells, the CEM-178 clone was 100-fold more resistant to growth inhibition induced by indanocine, with IC_{50}s of 1.79 and 206 nM, respectively (Table 2). The resistant phenotype persisted when the mutant cells were cultured in drug-free medium for at least 9 months. CEM-178 cells grew slightly slower than parental CEM cells, with doubling times of 24 and 18 h, respectively. Quantitative analyses of DNA content by flow cytometry, as well as karyotyping, showed no difference between CEM and CEM-178 (data not shown).
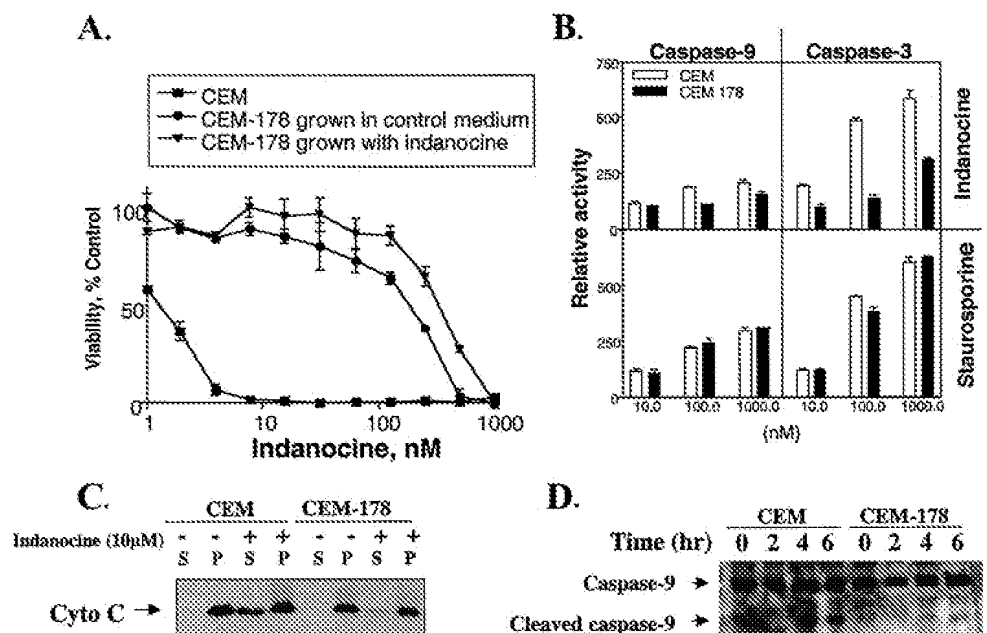
Compared with indanocine, the CEM-178 cells displayed reduced cross-resistance to two other inhibitors of tubulin polymerization, vinblastine (40-fold) and colchicine (31-fold; Table 2). In contrast, CEM-178 was not resistant to the tubulin-polymerizing drug paclitaxel, nor to the cytotoxic agents fludarabine, doxorubicin, and cytochalasin B. These results showed that CEM-178 is specifically resistant to microtubule-depolymerizing agents. Because paclitaxel is a substrate of gp170, the results also suggest that increased drug efflux may not contribute to the resistance. In fact, other experiments showed that the expression of the MDP gp170 was below detection levels in both parental CEM cells and the indanocine-resistant mutant (data not shown).

Most of the CEM cells were killed by ≤10 nM indanocine (48 h incubation), whereas CEM-178 cells remained viable after exposure to much higher concentrations (Fig. 1A). Indanocine kills an array of cell lines by inducing mitochondria dysfunction, cytochrome c re-

lease, caspase activation, and apoptosis (2). The drug failed to exert these effects in CEM-178 cells (Fig. 1, C and D), although the mutant retained functional apoptotic machinery as indicated by activation of caspases when treated with staurosporine (Fig. 1B). These results suggest that signaling event(s) upstream of cytochrome c release and caspase activation are altered in CEM-178 cells.

Effect of Indanocine on Tubulin Depolymerization. Immunoblotting data indicated that the expression levels of α-tubulin protein were similar between CEM and CEM-178 cells (Fig. 2A). To test the possibility that altered tubulin structure might confer indanocine resistance, we evaluated the effect of the drug on the relative levels of polymerized and soluble tubulin in parental and resistant cells. In untreated cells, >99% of the tubulin was in a free, soluble form (Fig. 2A). In unmanipulated cells, therefore, it was not possible to quantify an increased amount of soluble form induced by indanocine treatment. However, treatment with 5 μM paclitaxel for 1 h converted all of the tubulin in both CEM and CEM-178 cells into a polymerized form (Fig. 2A, + paclitaxel). On the basis of these observations, we pretreated the two cell lines with paclitaxel, washed out the drug, and then assayed the effect of indanocine exposure. CEM and CEM-178 cells were preincubated with 5 μM paclitaxel for 1 h, washed twice with drug-free medium, and then incubated with or without 10 μM of indanocine for 2 more h. After harvesting, soluble and polymerized tubulin were separated by centrifugation and assayed by immunoblotting. In wild-type CEM, ~50% of the polymerized tubulin underwent depolymerization after removal of paclitaxel (Fig. 2B, sample #1). In CEM-178 cells, the majority of tubulin remained in the pellet fraction, and no spontaneous depolymerization occurred (Fig. 2B, sample #3). When the paclitaxel-treated parental CEM cells were incubated with indanocine, almost all of the tubulin was converted to soluble forms (Fig. 2B, sample #2). In contrast, paclitaxel-pretreated CEM-178 cells were resistant to the depolymerization effects of indanocine, with >95% of tubulin remaining in the polymerized form (Fig. 2B, sample #4). To confirm visually the immunoblotting results, the treated cells were also stained with a specific anti-tubulin antibody. Parental CEM and CEM-178 displayed a similar network structure of microtubules after treatment with paclitaxel (Fig. 2C, panels 1 and 3). Indanocine induced a morphological change only in the parental CEM line, which displayed a diffuse pattern of microtubules, consistent with depoly-

Fig. 1. CEM-178 is resistant to indanocine-induced apoptosis. A, CEM wild-type (■), indanocine-resistant CEM-178 out of selection (●) and CEM-178 under selection with indanocine (▼) were treated with increasing concentrations of indanocine for 3 days. Viable cells were assessed by MTT assay. The results are expressed as the percentages of the control values without drug treatment. Bars, SD. B, CEM wild-type and CEM-178 were assayed for caspase activity after treatment with increasing concentrations of indanocine for 4 h and staurosporine for 16 h. Cells were lysed, and caspase activities were measured by cleavage of specific fluorimetric substrates for caspase-3 and caspase-9. Activities were represented in relative units to the control without treatment. Bars, SD. C, CEM and CEM-178 cells were each incubated with 10 μM of indanocine for 16 h. The cells were then fractionated into cytosolic and membrane-bound fractions and analyzed by Western blotting. After indanocine treatment, CEM wild-type cells released a significant amount of cytochrome c into the cytosol but CEM-178 did not. Cyto C, cytochrome C; S, sample; P, paclitaxel. D, CEM and CEM-178 cells were incubated with 10 μM indanocine for the indicated periods of time, lysed, and assayed for caspase-9 cleavage. Whereas indanocine induced caspase-9 cleavage at 4 h, no caspase-9 cleavage was observed for CEM-178.
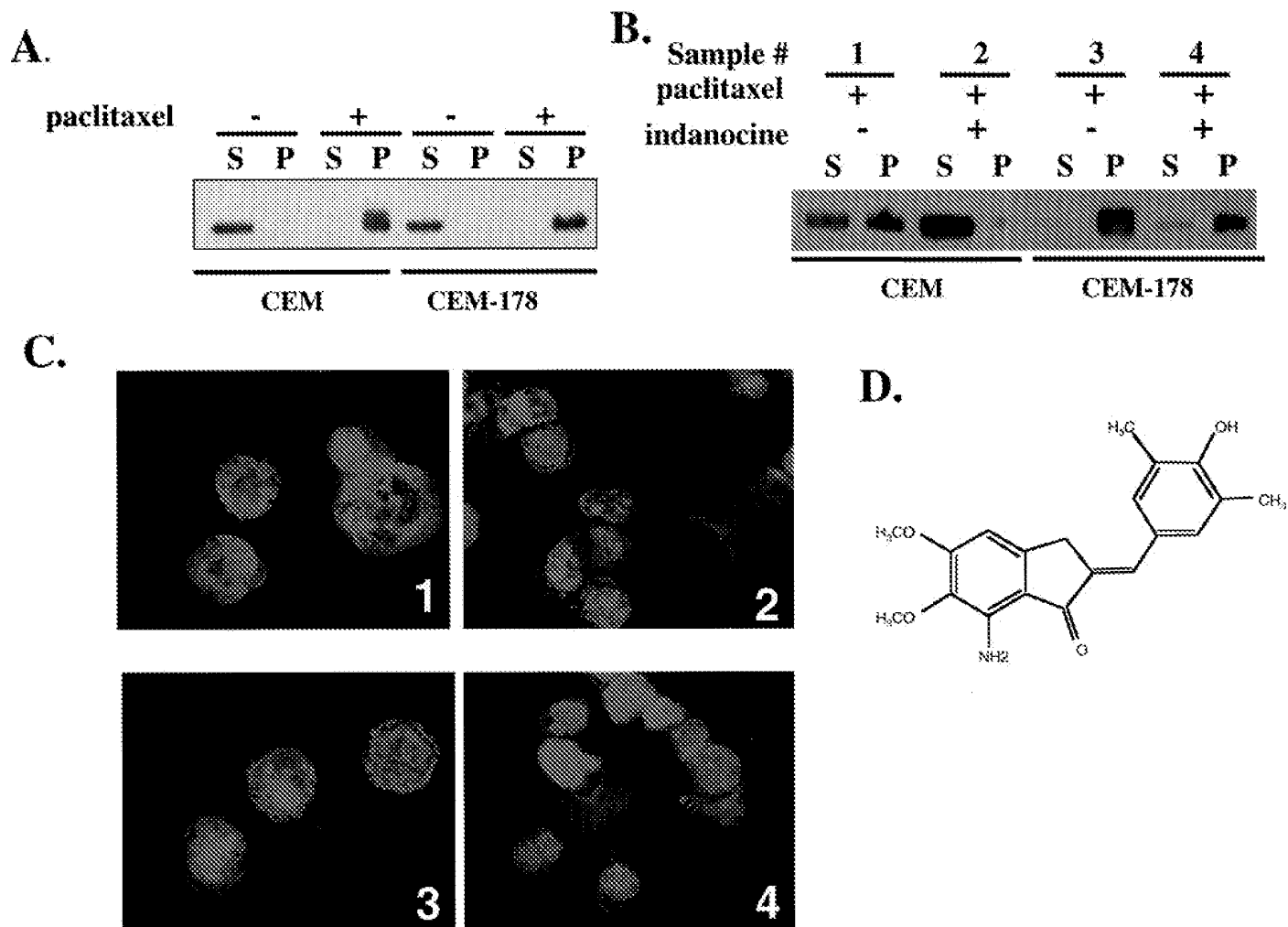

A.

CEM
CEM-178 grown in control medium
CEM-178 grown with indanocine

Viability, % Control

Indanocine, nM

B.

Caspase-9   Caspase-3

CEM
CEM 178

Relative activity

Indanocine

Staurosporine

(nM)

C.

CEM   CEM-178

Indanocine (10 μM)

S P S P S P S P

Cyto C →

D.

CEM   CEM-178

Time (hr) 0 2 4 6 0 2 4 6

Caspase-9 →

Cleaved caspase-9 →

Fig. 2. Indanocine induces tubulin depolymerization in CEM but not CEM-178 cells. *A*, CEM and CEM-178 cells were incubated in medium with or without paclitaxel for 1 h. In both cell types, tubulin existed in a free, cytosolic form. Paclitaxel converted tubulin into polymerized form. *S*, sample; *P*, paclitaxel. *B*, CEM and CEM-178 cells were treated with 5 μM of paclitaxel for 1 h and then washed twice with medium. The cells were then incubated with 10 μM indanocine for 2 h. Soluble and polymerized tubulin were assayed as described in "Materials and Methods." In wild-type CEM cells, polymerized tubulin underwent spontaneous depolymerization, and indanocine treatment completely depolymerized tubulin. In CEM-178 cells, there was no spontaneous depolymerization, and incubation with indanocine depolymerized only a small fraction of tubulin. *S*, sample; *P*, paclitaxel. *C*, CEM and CEM-178 cells were treated as in *B*. The cells were spun onto coverslips and stained for α-tubulin with a monoclonal antibody and with Alexa-568 conjugated secondary antibody (*red*). DNA was visualized with 4',6-diamidino-2-phenylindole (*blue*). Sample numbers correlate with those of *B*. *D*, structure of indanocine.

merization (Fig. 2*C, panel 2*). The CEM-178 line did not show any visible changes in the microtubule network (Fig. 2*C, panel 4*). Collectively, these results demonstrate that the microtubules in parental CEM and CEM-178 cells react differently to indanocine, and they are consistent with the hypothesis that there is a difference in tubulin structure between CEM and CEM-178 cells.

Cell-free Tubulin Polymerization Assays. To rule out the possibility that CEM-178 resistance might be attributable to a deficiency in drug uptake or active drug metabolism, tubulin depolymerization assays using a cell-free system were performed. Specifically, cells were incubated for 1 h in medium containing 5 μM paclitaxel, washed and lysed in tubulin extraction buffer, and incubated at 37°C for 2 h with 200 μM indanocine, followed by centrifugation and immunoblotting. In parental CEM cells, ~40% of the tubulin was depolymerized by indanocine (Fig. 3*A, CEM*). In contrast, almost all of the tubulin remained in a polymerized form in drug-resistant CEM-178 cells (Fig. 3*A, CEM-178*). It is important to emphasize that the tubulin extracted from CEM-178 cells did not lose its intrinsic ability to depolymerize, because incubation on ice induced a complete depolymerization (data not shown). Because incubating the cell lysate with indanocine made

the drug directly accessible to tubulin, this experiment eliminated the possibility that drug uptake or metabolism contributed to indanocine resistance. To determine whether a soluble factor required for tubulin depolymerization was missing or defective in CEM-178 cells, mixing experiments were carried out. After preincubation with paclitaxel, parental CEM and CEM-178 cells were lysed in tubulin extraction buffer containing indanocine. Then, the whole cell lysates from CEM cells were added to a CEM-178 lysate at ratios of 1:1, 1:5, and 1:10. The mixed lysates were incubated at 37°C for 2 h before tubulin depolymerization was assayed. Control experiments confirmed that indanocine induced tubulin depolymerization in the parental CEM lysate (Fig. 3*B, 1:0*) but not in the CEM-178 lysate (Fig. 3*B, 0:1*). When the lysates were mixed, the quantities of soluble tubulin decreased proportionally as the fraction of parental lysate decreased (Fig. 3*B, 1:1, 1:5, 1:10*). Thus, the indanocine-resistant cells did not contain a soluble inhibitor of tubulin polymerization.

Cell Fusion Studies. Cell fusion experiments between CEM-178 and an indanocine-sensitive CEM were carried out to confirm the cytosol mixing experiments. An ouabain-resistant, hypoxanthine phosphoribosyltransferase-negative CEM cell line was hybridized to
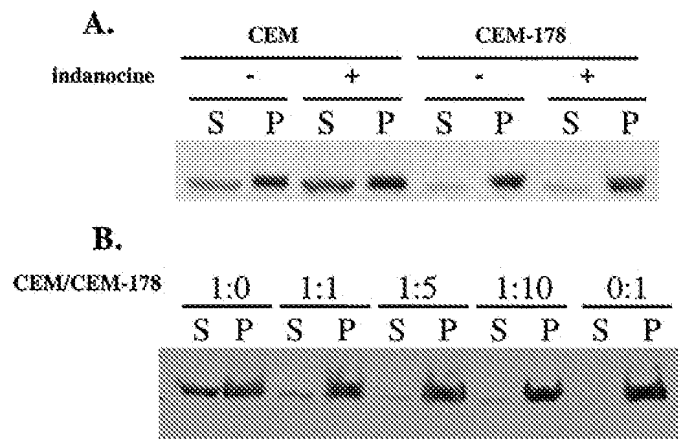
A.

B.

and b2, were sequenced in both directions in both parental CEM and CEM-178 cells. The cDNA sequence of the b2 isotype was identical in the two cell lines and was identical to the GenBank sequence (accession number X02344). In contrast, one point mutation was identified in the cDNA of the M40 isotype, with a G to T substitution at nucleotide 1050 (Fig. 5B), which converts amino acid 350 from lysine (AAG) to asparagine (AAU). The mutation was confirmed in two separate amplification and sequencing experiments. The M40 cDNA clone sequence in parental CEM cells was the same as that reported in GenBank (accession number J00314).

DISCUSSION

Indanocine selectively kills malignant B cells at doses that do not affect normal B cells. The CEM-178 mutant lymphoblastoid cell line is resistant to the toxic effects of the tubulin-binding drugs indanocine, vinblastine, and colchicine but is sensitive to paclitaxel. Consistent with this phenotype, CEM-178 cells do not overexpress the multidrug resistance-associated efflux pump Pgp170. Cells that overexpress Pgp170 are indanocine sensitive but paclitaxel resistant (2). Indanocine failed to depolymerize tubulin both in intact CEM-178 cells and in cell-free extracts. Data from extract mixing and cell fusion experiments ruled out the possibility of a missing soluble factor in CEM-178 that is required for indanocine killing but suggested instead that a structural change in tubulin conferred resistance to the drug. In support of this conclusion, a point mutation in β-tubulin, at amino acid 350, was detected in the indanocine-resistant CEM-178 mutant but not in parental cells. The mutation caused a lysine to arginine substitution in the β-tubulin molecule.

The pronounced effect of the point mutation on indanocine toxicity can be explained in at least two ways: (a) it is possible that positively charged Lys[350] represents an important interacting site for indanocine that is lost when changed to Asn; and (b) alternatively, it is possible that this mutation changes the overall organization of tubulin, making the microtubule network more rigid. As a result, higher concentrations
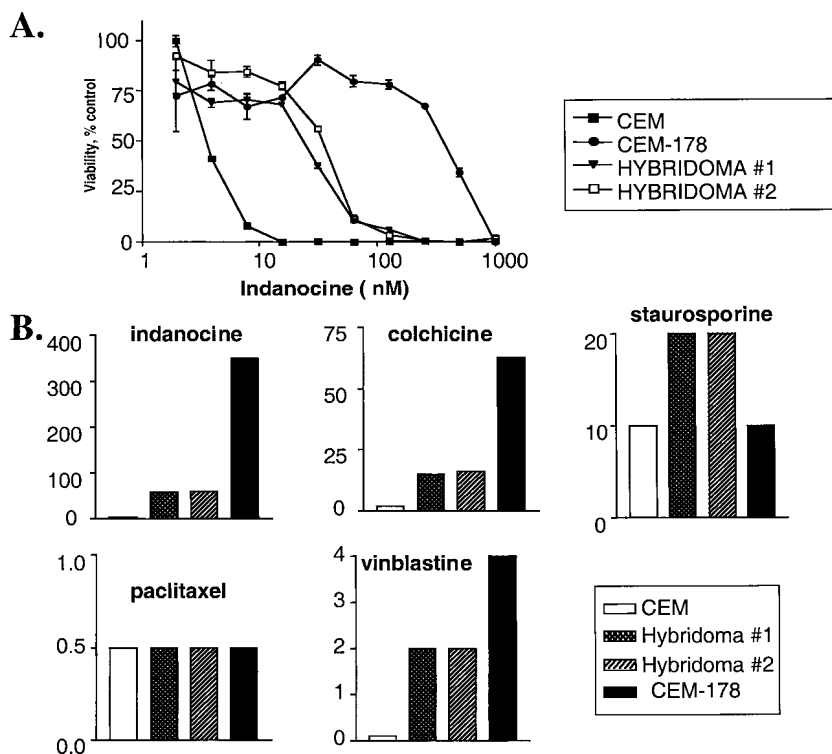
Fig. 3. Mixing experiments. *A*, parental CEM and CEM-178 cells were preincubated with 5 μM paclitaxel for 1 h. The cells were harvested and lysed in a buffer containing 200 μM indanocine and incubated for another 2 h. Then, cytosolic and polymerized tubulin were assayed. Indanocine depolymerized tubulin *in vitro* in CEM cells but not in CEM-178 cells. *S*, sample; *P*, paclitaxel. *B*, CEM and CEM-178 cells were pretreated with paclitaxel and lysed in tubulin extraction buffer with indanocine as in *A*. The lysate from parental CEM cells was mixed with that of CEM-178 at the indicated ratios. The mixed lysates were incubated at 37°C for 2 h and then centrifuged to separate soluble and polymerized tubulin. Tubulin content in each fraction was quantified by Western blotting with a specific anti-α-tubulin antibody. *S*, sample; *P*, paclitaxel.

CEM-178, and heterokaryons were selected and cloned in ouabain-HAT medium. The toxicity of indanocine to several hybridoma clones was between CEM and CEM-178 (Fig. 4A). Cross-resistance was partially maintained in the hybridomas (Fig. 4B) in that both vinblastine and colchicine were less toxic to the fused cells than to wild-type CEM. These results indicated that the resistance phenotype of the CEM-178 was retained in the fusion cells in a codominant fashion and suggested the possibility of a tubulin mutation.

Sequencing of β-Tubulin b2 and M40 Isotypes. To determine whether a mutation in tubulin was responsible for indanocine resistance, the cDNAs for the two predominant isotypes of β-tubulin, M40

A.

Fig. 4. Indanocine toxicity in hybridoma cell lines and cross-resistance to other drugs. *A*, wild-type CEM (■), indanocine-resistant CEM-178 (●), and two different hybridomas (▼, □) resulting from the fusion of CEM and CEM-178 were assayed for indanocine toxicity. *Bars*, SD. *B*, IC_{50} (in nM) for CEM, CEM-178, hybridoma #1, and hybridoma #2 were plotted for different drugs.
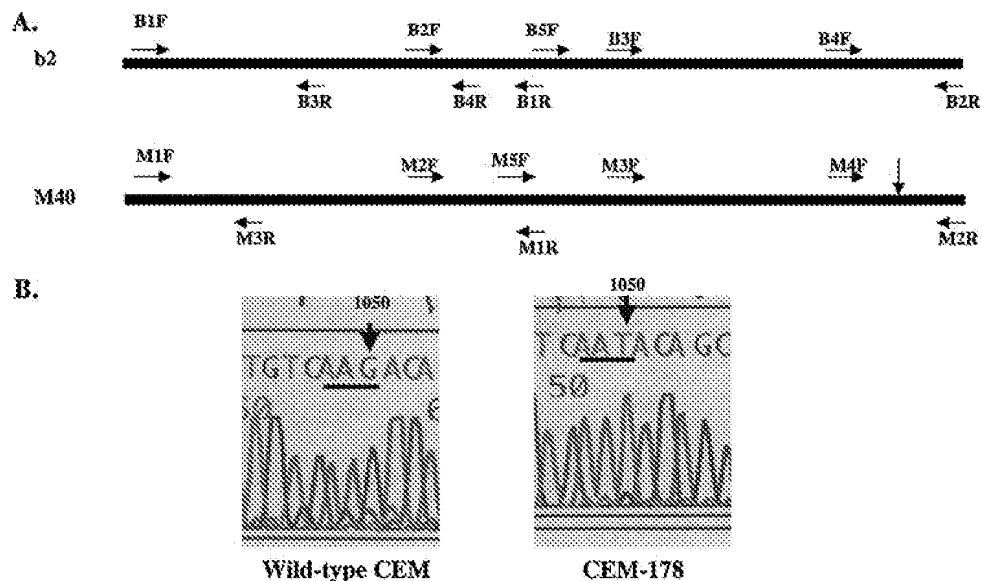
B.

Fig. 5. Sequencing of β-tubulin in CEM-178 reveals a point mutation in the M40 isotype. *A*, schematic diagram of primers used in sequencing of b2 and M40 tubulin. *Vertical arrow*, location of the point mutation. *B*, sequence readout showing that a G residue in position 1050 in wild-type CEM is converted to T in CEM-178.

of indanocine are required to depolymerize the microtubule and to induce cell death. We favor the first mechanism for the following reasons: (*a*) CEM-178 grew normally in regular medium, suggesting that the overall microtubule organization was adequate for rapid cell division; (*b*) Western blot analysis showed that in both CEM and CEM-178 cells, tubulin existed largely in a depolymerized form (Fig. 2*A*). No increased polymerized tubulin was observed in CEM-178; (*c*) if the mutation caused a more rigid microtubule network, it
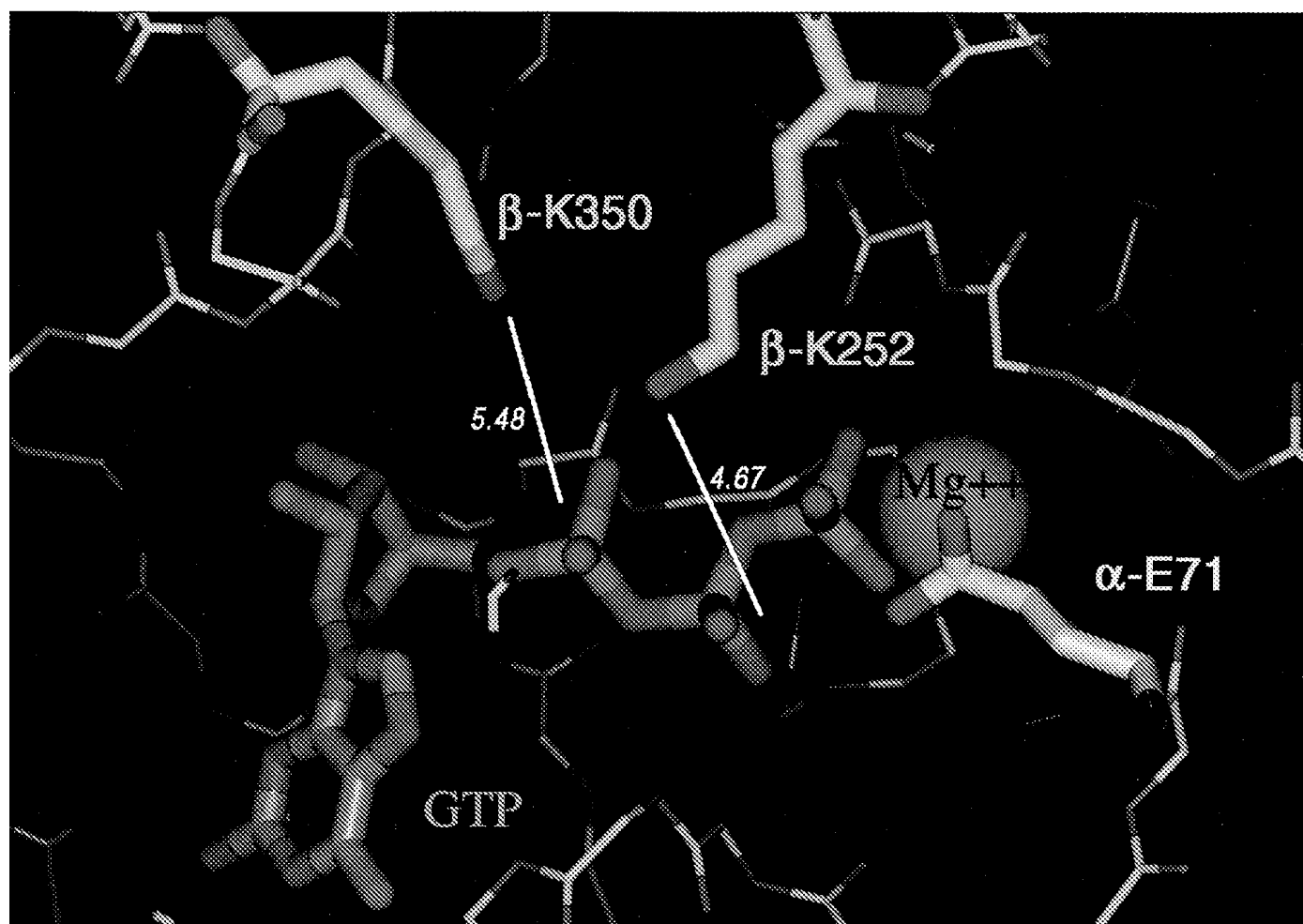

Fig. 6. Proposed electrostatic stabilization of the GTP phosphates by β-K350 of tubulin. The first phosphate is stabilized by the β-K350 (carbons in *cyan*) side chain, the second by β-K252 (carbons in *yellow*) at the shown distances. The terminal phosphate along with the carboxylate of α-E71 (carbons in *yellow*) is stabilized by a Mg^{2+}.

should have increased the threshold for all depolymerizing agents. Therefore, the resistant line should exhibit similar degrees of cross-resistance to other depolymerizing drugs. This is not the case, because CEM-178 is 115-fold more resistant to indanocine but only 40- and 31-fold resistant to vinblastine and colchicine, respectively. Preliminary experiments also have shown that tubulin depolymerization induced by cold temperature occurs at equivalent rates in CEM and the indanocine-resistant mutant (data not shown). Collectively, these results suggest strongly that the identified point mutation on β-tubulin represents a specific interaction site for indanocine.

Increasing evidence from several laboratories suggests that alterations in tubulin primary sequence can play a role in resistance to antimitotic agents. For example, mutations in $\beta270$ and $\beta364$ have been shown to confer paclitaxel resistance in human ovarian cancer cells (5). Cell lines resistant to epothilone A and B have mutations at $\beta274$ and $\beta282$ (6).

In contrast to the accumulating knowledge of the paclitaxel-binding site on β-tubulin, the binding sites for colchicine and vinblastine have not been mapped by mutational/molecular modeling approaches. By using light-activated colchicine analogues, Uppuluri et al. identified amino acids 214–241 as a probable site for colchicine interaction (11). Other indirect evidence suggests that Cys-354 and Cys-239 play a role in the colchicine binding site (12, 13).

Our results suggest that Lys^{350} might be an important interaction site for indanocine and colchicine. On the basis of the crystal structure of tubulin (14), the α carbon on β-K350 is located only 10 angstroms from the first phosphate group of GTP, which binds near the interface of the α and β monomers. Therefore, we investigated the possibility that β-K350 might provide some functional interaction with GTP, such as charge stabilization. For a fully charged nucleotide or charged amino acid side chain to exist deeply in the interior of a protein, there must be some form of neutralizing bridge. We first observed that the second phosphate group of GTP was already neutralized by the epsilon amino group of β-K252, which is only 4.67 angstroms away. Next, we found that the terminal phosphate of GTP is located very close (4.4 angstroms) to the carboxylate of α-E71 in the Nogales tubulin model (14). On the basis of this observation, as well as the fact that no other basic residues are in the vicinity of α-E71, we formed a model that demonstrates the possibility that a Mg^{2+} can bind and neutralize both α-E71-carboxylate and GTP-terminal phosphate anions. Finally, we were able to modify the dihedral angle of the β-K350, enabling its epsilon amino group to be within 5.48 angstroms from the first phosphate, a distance that favors a stable, neutralizing salt bridge between these two groups. On the basis of the results of these modeling studies, we propose that the β-K350, β-K252 side chains may serve to neutralize the charges of the first two phosphates

of GTP at the non-exchangeable site, and that the terminal phosphate along with the carboxylate of α-E71 charges are coneutralized by a Mg^{2+}. Fig. 6 illustrates the results of this model.

Taken together, the results reported in this study explain in part the molecular mechanism of action of indanocine and should facilitate the rational design of other tubulin-binding agents with anticancer activity.

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